Diabetes Mellitus Activates Signal Transduction Pathways Resulting in Vascular Endothelial Growth Factor Resistance of Human Monocytes

Vadim Tchaikovsky, MD; Servé Olieslagers, BSc; Frank-D. Böhmer, PhD; Johannes Waltenberger, MD, PhD

Background—Monocytes are cellular components of wound repair, arteriogenesis, and atherogenesis. Vascular endothelial growth factor (VEGF)-A and placental growth factor recruit monocytes to sites of arteriogenesis via stimulation of VEGF receptor-1 (VEGFR-1). The chemotactic response of monocytes to VEGF-A is attenuated in individuals with diabetes mellitus (DM). This VEGF resistance correlates with impaired collateral growth. The aim of this study is to elucidate the molecular basis of VEGF resistance and impaired monocyte response in DM.

Methods and Results—Phosphorylation of Akt, p38, and extracellular signal-regulated kinase 1/2 (ERK1/2) could be stimulated with either placental growth factor-1 or VEGF-A in monocytes from non-DM but not DM individuals. In contrast, formyl-methionyl-leucyl-phenylalanine caused a comparable activation of these molecules in both DM and non-DM monocytes. Baseline phosphorylation of Akt, p38, and ERK1/2 was significantly elevated in monocytes from DM compared with non-DM subjects. Of note, H2O2 activated Akt, p38, and ERK1/2 in non-DM monocytes but not DM individuals, which reflects functional protein tyrosine phosphatase inhibition, similar to that seen after H2O2 challenge. Overall, protein tyrosine phosphatase and protein tyrosine phosphatase-1B activity were reduced in DM monocytes. DM monocytes revealed higher expression of the receptor for advanced glycation end products. Stimulation with advanced glycation end products ligands resulted in activation of non-DM monocytes and inhibition of VEGFR-1–mediated chemotaxis. The elevated baseline phosphorylation/activation of Akt, p38, and ERK1/2 in DM monocytes likely causes the resistance to further stimulation with specific stimuli such as VEGF-A, revealing a molecular explanation of the DM-related signal transduction defect.

Conclusions—We propose that elevated advanced glycation end products expression and increased oxidative stress in diabetic monocytes lead to activation of VEGFR-1–related signaling pathways and to desensitization of VEGFR-1 responses. These data establish VEGF resistance as a novel molecular concept for DM-related cellular dysfunction. (Circulation. 2009;120:150-159.)

Key Words: diabetes mellitus ▪ monocytes ▪ signal transduction ▪ vascular endothelial growth factor receptor-1

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Growth factors are potent and crucial mediators of vascular growth processes, including angiogenesis and arteriogenesis.1,2 Both processes can enhance regional blood flow and restore impaired tissue function. Monocytes contribute to arteriogenesis by recruitment to the growing vessel wall.3–5 Moreover, angiogenesis is monocyte dependent in the context of wound healing.6

Critical Perspective on p 159

The family of vascular endothelial growth factor (VEGF) and its receptors (VEGFR) are mediators of angiogenesis and arteriogenesis in both embryonic development and adult life.1,4–7 VEGF-2 mediates crucial functions of endothelial cells such as proliferation, migration, nitric oxide synthase upregulation, nitric oxide release, and survival.8–10 However, the role of VEGFR-1 in endothelial cells is less clear, although it can cross-talk with VEGFR-2, which is required for maximal activation of VEGFR-2.11,12 The proven function of VEGFR-1 in adult life is its chemotactic action on monocytes and macrophages.13–15 Likewise, cytokine (tumor necrosis factor-α and interleukin-1β) and chemokine (monocyte chemotactic protein-1, interleukin-8, and macrophage inflammatory protein-1β) gene expression and chemotaxis are VEGFR-1 dependent in the monocytic cell line THP-1.16
Among cardiovascular risk factors, diabetes mellitus (DM) is the most important one that negatively affects vascular cell function, the integrity of the arterial wall, and the growth of new vessels. The development and function of coronary collateral vessels are significantly reduced in patients with DM. The reduced collateral growth in diabetic individuals corresponds with a significantly altered chemotactic response of monocytes to VEGF-A ex vivo (VEGF resistance), which is associated with impaired arteriogenesis. An intracellular signal transduction defect downstream from VEGFR-1 was suggested as the cause of the impaired monocyteic response, although the tyrosine kinase function of VEGFR-1 remained intact in monocytes derived from diabetic individuals.

DM-associated hyperglycemia is a risk factor for both microvascular and macrovascular complications. Persisting hyperglycemia can cause long-term alterations in cellular biochemistry by inducing production of reactive oxygen species (ROS) and excessive formation of advanced glycation end products (AGEs). These glucose-induced biochemical alterations adversely affect cellular function by altering various signal transduction pathways. The underlying molecular basis remains largely unclear; however, they are likely to be the result of negative pathological alterations, including oxidative stress (reviewed elsewhere).

Monocyte chemotaxis in response to ligand-induced VEGFR-1 activation is mediated by distinct signal transduction pathways involving PI3K/Akt or the mitogen-activated protein kinases p38 and extracellular signal-regulated kinase 1/2 (ERK1/2). The activation of these pathways is initiated by upstream kinases such as the VEGFR-1 tyrosine kinase, whereas their inactivation occurs as a result of functional counteraction by protein tyrosine phosphatases (PTPs). This reflects a balance between tyrosine kinases and PTPs.

In the present study, we show for the first time that monocytes from DM individuals display an increased baseline activation of VEGFR-1–related signal transduction pathways. As a consequence, the VEGFR-1 ligands placental growth factor (PIGF)-1 and VEGF-A are unable to further stimulate both activation and migration of monocytes from DM subjects. Oxidative stress in vitro causes increased baseline tyrosine phosphorylation in monocytes. Furthermore, oxidative stress is capable of inducing oxidative modifications of PTPs in monocytes from DM individuals, resulting in the inhibition of PTPs. These DM-related signaling defects represent a molecular basis for DM-related VEGF resistance of monocytes.

Methods
Characterization of Patient Groups
For a detailed description of materials and methods, see the online-only Data Supplement. The present study conforms to the principles of the Declaration of Helsinki. The study protocol was approved by the ethics committee of the University Hospital of Maastricht (the Netherlands). Patients with DM type 2 (DM group) and matched nondiabetic individuals (non-DM group) were studied (the Table). Patients with underlying inflammatory or malignant disease were excluded from the study. The 2 study groups showed similar characteristics and differed significantly only with regard to the use of calcium antagonists and statins and history of myocardial infarction. Informed consent was obtained according to the requirements of the local ethics committee. Patient enrollment in the study was performed in pairs; ie, on each occasion, an individual from the non-DM group was recruited together with an individual from the DM group. The subsequent handling of samples was performed in parallel.

Table. Characteristics of the Study Population

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<thead>
<tr>
<th>Measurement</th>
<th>Non-DM</th>
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<th>P</th>
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<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>Age, y</td>
<td>65.0 (56.0–73.0)</td>
<td>74.0 (61.0–77.5)</td>
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<td>Sex, M/F</td>
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<td>10/7</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>26.5 (24.0–30.75)</td>
<td>27.0 (25.0–29.0)</td>
<td>0.911</td>
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<tr>
<td>HbA₁c, %</td>
<td>ND</td>
<td>6.2 (6.9–8.1)</td>
<td>⋯</td>
</tr>
<tr>
<td>Glucose (at the time of blood sampling), mmol/L</td>
<td>5.4 (4.7–5.7)</td>
<td>8.6 (7.7–11.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Insulin (at the time of blood sampling), μIU/L</td>
<td>6.7 (6.0–16.5)</td>
<td>28.2 (10.5–49.8)</td>
<td>0.006</td>
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<tr>
<td>C-peptide (at the time of blood sampling), nmol/L</td>
<td>0.94 (0.74–1.36)</td>
<td>1.22 (0.95–1.65)</td>
<td>0.218</td>
</tr>
<tr>
<td>Medications, n</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACE inhibitors</td>
<td>6</td>
<td>5</td>
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<tr>
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<tr>
<td>Glycosides</td>
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<td>0</td>
<td>0.151</td>
</tr>
<tr>
<td>Statins</td>
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<td>16</td>
<td>0.003</td>
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<tr>
<td>Nitrates (long-acting)</td>
<td>5</td>
<td>8</td>
<td>0.297</td>
</tr>
<tr>
<td>Smoking (present/history), n</td>
<td>2/8</td>
<td>1/9</td>
<td>0.551/0.735</td>
</tr>
<tr>
<td>Coronary artery disease (stable/history of myocardial infarction), n</td>
<td>8/1</td>
<td>11/6</td>
<td>0.307/0.037</td>
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</tbody>
</table>

ACE indicates angiotensin-converting enzyme; AT-II, angiotensin type II.

Statistical Analysis
Statistical analysis was performed with SPSS 12.0.1 software (SPSS Inc, Chicago, Ill). The probability of differences between samples was evaluated with the Kruskal-Wallis test. Wilcoxon signed-rank test was used for intragroup analysis, and the Mann–Whitney test was used for intergroup analysis. Differences with values of P<0.05 were considered significant.

Results
Elevated Baseline Phosphorylation of Signaling Molecules in DM
We compared the activation and phosphorylation of distinct VEGFR-1–related signaling pathways in monocytes from DM and non-DM individuals (the Table). PIGF-1 stimulated chemotaxis of monocytes from non-DM but not from DM individuals (Figure 1A). Stimulation of monocytes from
non-DM individuals with either PlGF-1 or VEGF-A resulted in a significant increase in phosphorylation of Akt (1.53-fold and 2.54-fold, respectively; Figure 1C and 1E), ERK1 (2.13-fold and 2.26-fold, respectively; Figure 1C and 1G), and p38 (1.51-fold and 1.8-fold, respectively; Figure 1C and 1F) compared with baseline control values. Rather unexpectedly, DM monocytes did not respond to the stimulation with PlGF-1 or VEGF-A because these ligands could not stimulate phosphorylation of Akt, ERK1, or p38; their phosphorylation level remained at the level of the baseline controls in DM monocytes (Figure 1C). However, baseline values of Akt, ERK1, and p38 phosphorylation were significantly higher in the DM group compared with the non-DM group (Figure 1C and 1D): Akt baseline phosphorylation in DM was 2.95-fold higher than Akt baseline (referred to as 100%) phosphorylation in non-DM, whereas ERK1 showed a 2.06-fold and p38 a 2.51-fold increase in DM. Of note, these elevated baseline phosphorylation levels in DM monocytes were at, or even above, the levels reached for PlGF-1– or VEGF-A–induced phosphorylation of Akt, ERK1, or p38 in non-DM monocytes.

The level of baseline activation/phosphorylation in DM individuals correlated with the hemoglobin A1c (HbA1c) level. When DM individuals were split into 2 groups, ie, with HbA1c levels above and below the median value (6.9%), ERK1/2 baseline phosphorylation was significantly higher in the subgroup with HbA1c levels above the median (P<0.05; Figure 2C). Baseline phosphorylation of neither Akt (P>0.05; Figure 2A) nor p38 (P>0.05; Figure 2B) showed any significant differences between the 2 subgroups.

Is this DM-related defect specific for VEGFR-1–mediated signals? This seems to be the case, at least to some extent. Formyl-methionyl-leucyl-phenylalanine (fMLP), which induces monocyte migration in both non-DM and DM monocytes (Figure 3A and elsewhere), was capable of inducing significant activation of several signaling molecules in cells from both non-DM and DM individuals. fMLP stimulation increased Akt phosphorylation 6.97-fold in non-DM and 7.27-fold in DM (Figure 3B and 3C); p38 phosphorylation increased to 4.72-fold in non-DM and to 2.58-fold in DM (Figure 3B and 3D); and ERK1 phosphorylation increased 7.72-fold in non-DM and 3.04-fold in DM monocytes (Figure 3B and 3E). The magnitude of fMLP-induced phosphorylation was not significantly different between non-DM and DM monocytes (Figure 3C through 3E). fMLP was readily...
able to induce phosphorylation of Akt, ERK1, and p38 in DM monocytes despite the elevated baseline phosphorylation (Figure 3B).

Increased Oxidation of PTPs in Monocytes From DM Individuals In Vivo and Under Oxidative Stress In Vitro
Monocytes from DM individuals showed increased levels of reversible PTP oxidation compared with non-DM monocytes in the modified in-gel assay (Figure 4A). This is reflected by a higher PTP activity of certain PTP species (indicated in Figure 4A) in DM monocytes recovered in the presence of sodium iodoacetate. The identity of these PTPs remains to be established. These data clearly indicate a DM-related deregulation and oxidation of PTPs.

Incubation of non-DM monocytes with H2O2 caused oxidative stress in vitro. Increasing H2O2 concentrations augmented reversible oxidative modifications of PTPs (Figure 4B).

PTP Activity Is Reduced in DM Monocytes
Monocytes from DM individuals showed a significantly decreased overall PTP activity compared with non-DM monocytes. This was measured by the release of free phosphate from a prelabeled substrate (Figure 4C). Likewise, the specific PTP1B activity tended to be reduced in DM monocytes (Figure 4D) compared with their non-DM counterparts.

PTP Inhibition in Monocytes In Vitro Results in Activation of Kinases and Inhibition of Motility
Incubation of monocytes from non-DM individuals with the PTP inhibitor bpV(phen) led to a strong activation of ERK1/2 (Figure 4E) in a time- and dose-dependent manner. P38 activation appeared in a somewhat delayed fashion. Increased Akt phosphorylation was observed only at higher PTP inhibitor concentration. We preincubated monocytes from non-DM individuals with different concentrations of bpV-(phen) and analyzed their chemotactic responses to PIGF-1, VEGF-A, or fMLP (Figure 4F). The PTP inhibitor caused a dose-dependent decrease in chemokinesis and chemotaxis toward PIGF-1 and VEGF-A. Therefore, the biochemical and functional consequences of bpV(phen) pretreatment are similar to those detected in DM monocytes.

Oxidative Stress Activates Monocyte Signaling
Incubation of non-DM monocytes with H2O2 in vitro resulted in a strong, although distinct, increase in phosphorylation of Akt, p38, and ERK1/2 (Figure 5). Both 0.1 and 1 mmol/L H2O2 caused phosphorylation of Akt, p38, and ERK1/2 after already 1 minute of incubation (Figure 5A). The level of ERK1/2 phosphorylation further increased tremendously after 5 and 25 minutes, whereas Akt phosphorylation reached a maximum after 5 minutes of incubation with H2O2. Prolonged 25-minute incubation resulted in an additional increase in ERK1/2 phosphorylation: The H2O2-induced phosphorylation of p38 reached its maximum level earlier, ie, after 1 minute of incubation (Figure 5A).

To directly compare the effect of different concentrations of H2O2 on enzyme phosphorylation, a 5-minute incubation period was used (Figure 5B). Akt phosphorylation was already increased at 0.001 mmol/L H2O2, reaching its maximum at 1 mmol/L. Phosphorylation of ERK2 greatly increased at 0.1 to 1 mmol/L H2O2. A similar pattern was observed for ERK1 with a slight initial increase at 0.01 mmol/L H2O2. Phosphorylation of p38 rose drastically over the unstimulated level at a concentration of 0.1 mmol/L H2O2, with a further increase at 1 mmol/L.

Receptor for Advanced Glycation End Products Is Expressed in Monocytes and Mediates Monocyte Activation
Do primary monocytes express the functional receptor for AGE (RAGE)? We addressed this important question. Monocytes from non-DM individuals expressed full RAGE and both C-truncated and N-truncated RAGE splice variants (Figure 6A). Furthermore, primer sets designed to specifically amplify multiple splice variants (full length and N truncated or full length and N and C truncated) generated the expected amplicons (Figure 6A).

The presence of RAGE protein in primary human monocytes from non-DM individuals was confirmed by fluores-
cence-activated cell sorter analysis using an antibody against the C terminus of RAGE, therefore recognizing only full-length and N-truncated RAGE. CD14/H11001 monocytes expressed RAGE. Critically, 2 subpopulations of CD14/H11001 monocytes could be distinguished with different levels of RAGE expression (Figure 6B). Of note and in line with our signaling data (above), monocytes from DM individuals expressed higher levels of RAGE protein (range, 1.31- to 2.17-fold) compared with non-DM monocytes (Figure 6C, top). Full-length RAGE appeared as a 50-kDa band (Figure 6C), similar to the observations in primary endothelial cells and pericytes.24 Can RAGE stimulate the activation of primary monocytes? RAGE ligation with its ligand S100b at 1 and 10 μg/mL led to the phosphorylation of Akt and ERK1/2 (Figure 6D). Akt phosphorylation (Figure 6D, top) was maximal at 5 to 15 minutes after RAGE stimulation, whereas ERK1/2 phosphorylation (Figure 6D, middle) peaked after 45 minutes. Phosphorylation of p38 (Figure 6D, bottom) remained unaffected by RAGE stimulation.

We investigated whether RAGE activation in non-DM monocytes affects their chemotactic response. S100b (10 μmol/L) leads to Akt and ERK1/2 phosphorylation (Figure 6D). Preincubation of monocytes with S100b (10 μg/mL for 1 hour) inhibited monocyte chemotaxis to PlGF-1 and VEGF-A but not to fMLP (Figure 6E). These data support the contribution of RAGE ligation in monocyte activation in DM.

**Discussion**

This is the first report on molecular mechanisms of growth factor signal transduction defects in primary human monocytes. This aspect is of major pathophysiological importance because monocytes have recently been established as important cellular mediators of vascular growth, including arteriogenesis4,5 and wound healing.6 Moreover, monocyte dysfunction has been described in the context of DM.19 We now report that type 2 DM–related alterations of the activation of cellular signaling pathways, including Akt, p38, and ERK1/2, contribute to the observed VEGF resistance, namely the blunted monocyte responsiveness to VEGFR-1–mediated growth factor stimulation. The elevated baseline activity of these pathways in DM results in monocyte activation and refractoriness to further specific stimulation. It may be caused by the increased production of ROS (oxidative stress), which inhibits PTPs, and/or by increased signaling via the AGE/RAGE axis. It remains to be clarified whether the 2 pathways operate independently or cooperate (Figure 7).
An important physiological function of monocytes is chemotaxis (ie, their ability to migrate along gradients of growth factors and cytokines). Monocyte migration to sites of vascular growth is VEGFR-1 dependent. Recent evidence suggests that DM and its underlying pathophysiological mechanisms affect the function of circulating cells with angiogenic or arteriogenic potential. The chemotactic response of monocytes to VEGF-A19 or PlGF-1, ligands of VEGFR-1 (Figure 1A), was completely blunted in DM monocytes but remained intact toward the (strong) inflammatory mediator fMLP (Figure 3A and elsewhere). This functional defect of DM monocytes occurs despite unaffected expression (Figure 1B) and preserved function of VEGFR-1. Likewise, CD34+ cells derived from DM subjects also showed a functional migratory defect in response to stromal cell–derived factor-1. Similarly, transendothelial migration of DM monocytes toward the proinflammatory chemokines CCL2 and CCL3 was decreased.

The adverse effect of acute hyperglycemia on the activation of particular signaling pathways in circulating cells has recently been established. In monocytes, type 2 DM was associated with increased expression of the glucose-sensitive isoform of protein kinase C, which was activated. Likewise, MEK/ERK1/2 pathway activation was demonstrated in monocytes of healthy volunteers subjected to acute hyperglycemia. Similarly, CD14+ monocytes from DM individuals were found in an activated state with increased expression of several proinflammatory genes. Mononuclear cells, when cultured under hyperglycemic conditions, revealed notable increases in the expression of several transcription factors. The impaired cellular physiology was due to activation of the p38 signaling pathway; specific blockade of this signaling pathway reversed the hyperglycemia-related impairment of cell function.

As shown recently, distinct signal transduction pathways, including p38, ERK1/2, and Akt, mediate monocyte migration in response to VEGFR-1 activation. Our present data demonstrate that these signaling pathways cannot be further activated in DM monocytes on stimulation with VEGFR-1 ligands. Therefore, DM-related preactivation of monocyte signaling pathways represents the molecular basis for the...
refractoriness of diabetic monocytes to this specific, VEGFR-
1–mediated chemotactic stimulus reflecting VEGF resistance.

Several studies have reported increased levels of AGEs in
DM populations.23 Repeatedly elevated glucose plasma levels
result in the glycation of proteins and the formation of AGEs,
including HbA1c.23 RAGE is a functional receptor, and
AGE-RAGE interaction leads to the activation of distinct
signaling pathways for p38 and ERK1/2 in the monocytic cell
line THP-1.32 This is associated with a proinflammatory
acellular activation with increased COX-2 mRNA and prosta-
glandin E2 expression.32 The existence of different splice
variants of RAGE in endothelial cells has recently been
reported.33 For the first time, we report here the presence of
all 3 splice variants of RAGE, ie, C-truncated, N-truncated,
and full-length variants, in primary human monocytes. Their
individual contribution to cardiovascular pathology and DM
remains to be clarified.34,35

We report an increased expression of full-length RAGE
(protein) in DM monocytes (Figure 6C). This observation
may have critical implications for the pathophysiology
of DM, considering that RAGE ligands upregulate RAGE ex-
pression.23 Furthermore, ligation of RAGE with S100b, an
established ligand of RAGE,32 caused an increase in Akt and
ERK1/2 phosphorylation in monocytes from non-DM sub-
jects.
mechanism negatively regulating PTP activity. In contrast, oxidation of catalytic cysteine, is an established physiological

HbA1c is used primarily to identify the average plasma

site. Reversible oxidation of PTPs, involving targeted

the reduced status of a cysteine sulfhydryl group in the active

PTPs. The proper function of phosphatases is dependent on

signal transduction involve protein dephosphorylation by

PTPs, mediating monocyte migration (non-DM; thick black arrow). In DM, elevated RAGE expression and signaling in monocytes leads to increased baseline signaling activity. Furthermore, oxidative stress (ROS) may contribute either directly to the increase in baseline signaling activity or indirectly via inhibition of PTPs. The increase in ROS is attributable mostly to hyperglycemia (solid arrow). It is possible that RAGE activation also contributes to ROS production and PTP inactivation; however, such links have yet to be established (dashed arrows). The increased baseline signaling activity results in desensitization of VEGFR1 responses (DM; thin black arrow).

jects (Figure 6D). This observation is in line with the significantly elevated baseline phosphorylation level of ERK1/2 in the subgroup of DM individuals with high HbA1c values (Figure 2A). Baseline phosphorylation of Akt also showed a trend to be increased in the subgroup with high HbA1c levels (Figure 2B) without notable changes for p38. HbA1c is used primarily to identify the average plasma glucose concentration over prolonged time periods. We therefore propose that baseline ERK1/2 phosphorylation in monocytes may serve as a marker for DM-related cellular dysfunction and may prospectively be used to monitor cellular (dys)function in DM. Moreover, we studied the effect of S100b on monocyte motility in vitro to model elevated AGE levels in DM. Preactivation of nondiabetic monocytes with S100b inhibits VEGFR-1-mediated chemotaxis (Figure 6E), resembling the diabetic phenotype. Together, these findings highlight the functional role of RAGE in DM-induced monocyte activation.

Oxidative stress has been implicated in the pathophysiology of diabetic complications. Monocytes from DM individuals produce significantly more superoxide O₂⁻, This observation was causally linked to RAGE ligation on THP-1 cells. We now show that oxidative stress in vitro leads to the activation of Akt, p38, and ERK1/2 in non-DM monocytes in a dose- and time-dependent manner (Figure 5).

The activation of kinases occurs via phosphorylation of target proteins, whereas inactivation and termination of signal transduction involve protein dephosphorylation by PTPs. The proper function of phosphatases is dependent on the reduced status of a cysteine sulfhydryl group in the active site. Reversible oxidation of PTPs, involving targeted oxidation of catalytic cysteine, is an established physiological mechanism negatively regulating PTP activity. In contrast, prolonged oxidative stress under pathological conditions such as DM may even result in irreversible oxidation of PTP. Such a modification of PTPs resulting in their functional knock-down was reported under hyperglycemic conditions in vitro. Oxidized cysteine cannot function as a phosphate acceptor, a crucial step in PTP function. We now report that particular PTPs in DM monocytes have increased reversible oxidative modifications. This deregulation can potentially explain their malfunction. We observed similar effects on PTPs in monocytes under conditions of oxidative stress in vitro. In addition, we report here for the first time that overall PTP activity is decreased in DM monocytes, as is the activity of the phosphatase PTP1B. These findings provide initial evidence that increased generation of ROS in DM may lead to functional downregulation of PTPs and thereby contribute to kinase activation. Moreover, we demonstrated for the first time that the inhibition of PTP with bpV(phen) not only activated some intracellular signaling cascades (including of Akt, ERK1/2, and p38) but also inhibited chemokinesis and VEGFR-1-mediated chemotaxis. These consequences of PTP inhibition are essentially similar to the biochemical and functional consequences observed in diabetic monocytes and thereby strongly implementing oxidation-related impairment of PTP function as a mechanism contributing to the signal transduction defect in DM individuals.

There is accumulating evidence that circulating cells with angiogenic potential, namely monocytes and progenitor cells, are adversely affected under diabetic conditions. This results in an aberrant ability of these cells to adhere to or to migrate toward specific stimuli such as VEGF-A. The same is true for cellular homing to sites of angiogenesis. Our data suggest that the DM-associated impairment of VEGFR-1-mediated monocyte chemotaxis is caused by the preactivation of critical signal transduction pathways. This activation disables monocytes from responding to further stimulation with (among others) VEGF-1 ligands. The reported monocyte preactivation appears to involve signaling through RAGE and increased production of ROS, both well-established pathophysiological mechanisms of DM. Increased levels of ROS are likely to be the result of hyperglycemia. It remains to be established whether the RAGE-mediated augmentation of ROS production (as demonstrated in THP-1 cells) has implications for monocyte dysfunction in DM (Figure 7, dashed line). Increased levels of ROS may lead to the direct activation of signaling molecules/kinases in monocytes. Simultaneously, increased levels of ROS may indirectly lead to kinase activation (or decreased inactivation) by inhibiting PTP activity by means of oxidation. We identified PTP1B as a candidate PTP, which is inhibited in DM monocytes. Our observations provide a novel molecular basis to explain the DM-related signal transduction defect resulting in monocyte dysfunction.

Our data describe a novel pattern of DM-related molecular alterations in monocytes. These include oxidative stress and RAGE signaling, both leading to nonspecific monocyte activation with a functional negative impact on consecutive monocyte activation via VEGFR-1 (Figure 7) and resulting in VEGF resistance. Selective alteration of VEGFR-1 signaling (in contrast to fMLP-induced signaling) in DM indicates a
higher sensitivity, ie, vulnerability of the VEGFR-1 system. The described alterations related to DM-induced monocyte dysfunction may represent a basis for the therapeutic correction of DM-related cellular dysfunction by interfering on the level of molecular oxidation and/or glycation. Furthermore, these data demonstrate that circulating cells may function as “biosensors,” translating metabolic aberrations into altered cellular function.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

This article describes a functionally relevant molecular defect in monocytes from individuals with diabetes mellitus (DM). It is well established that monocytes play a crucial role in both arteriogenesis (ie, collateral growth) and atherogenesis. Moreover, there is evidence that DM-related monocyte dysfunction such as impaired monocyte chemotaxis toward vascular endothelial growth factor-A (VEGF) is associated with impaired collateral growth and accelerated atherogenesis. We now report that the functional monocyte defect in DM is based on VEGF resistance: Although VEGF can activate its specific receptor, the migratory response is blocked. On a molecular level, this blockade/resistance can be explained by activation of crucial VEGF signaling pathways, including the Akt and mitogen-activated protein kinase pathways. DM is associated with reduced activity of protein tyrosine phosphatases, which allows enhanced activation of related kinases and results in nonspecific preactivation of relevant signaling pathways. In fact, we can mimic this diabetic phenotype by experimental inhibition of protein tyrosine phosphatases, again resulting in preactivation of signaling molecules and impaired chemotaxis. Likewise, receptor for advanced glycation end products activation, also known to occur in DM, results in similar changes associated with VEGF resistance. The clinical perspective of this concept is to use VEGF resistance and its underlying molecular changes for diagnostic purposes and thereby predict the efficiency of VEGF action in cardiovascular repair and potentially in therapeutic angiogenesis. Moreover, the therapeutic prevention of VEGF resistance could represent a novel concept to attenuate the negative functional consequences of DM in the vasculature.
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Manuscript: Diabetes mellitus activates signal transduction pathways resulting in VEGF resistance of human monocytes

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SUPPLEMENTAL MATERIAL

Materials and methods
Reagents
Recombinant human VEGF-A\textsubscript{165} was kindly provided by Denis Gospodarowicz (Chiron, Emeryville, CA, USA). PlGF-1 and VEGFR-1 antibody were obtained from RELIATech GmbH (Braunschweig; Germany) and formylMetLeuPhe (fMLP) was from Sigma-Aldrich (Saint Louis, Missouri; USA). Antibodies recognizing phosphorylated Akt (Ser473), ERK1/2 (Thr202/Tyr204) and p38 (Thr180/Tyr182) were from New England BioLabs/Cell Signaling (Beverly, MA; USA) and an anti-p38 kinase antibody was from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). Anti-RAGE antibody was from Chemicon (Temecula, CA, USA). Secondary horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit and donkey anti-goat antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). RPMI 1640 medium was purchased from Invitrogen (Karlsruhe; Germany). Fetal calf serum (FCS) was from Biochrom AG (Berlin, Germany). Western blot stripping buffer was from Pierce (Rochford, IL, USA). S100b and bpV(phen) were from Calbiochem (San Diego, CA, USA). Protein assay was from Bio-
Rad Laboratories GmbH (Munich, Germany) and Phosphatase Assay System and Cell Titer 96 Non-Radiactive Cell Proliferation Assay from Promega (Madison, WI, USA). Percoll separation solution was from Sigma-Aldrich (Saint Louis, MO, USA). Primers for RAGE were purchased from Eurogentec S.A. (Seraing, Belgium). All other chemicals were minimally of analytical grade.

Isolation of monocytes from peripheral venous blood
Monocytes were isolated from 100 mL of heparinized venous blood samples as described. In brief, density centrifugation was performed using the Ficoll separation solution with a density of 1.077 g/mL (Sigma-Aldrich) to isolate mononuclear cells. In a second round of centrifugation, monocytes were enriched using Percoll separation solution with a density of 1.139 g/mL (Sigma-Aldrich) before washing and resuspending the cells in RPMI 1640 (Invitrogen). The purity of the extracted monocytes was determined by analysis with a fluorescence-activated cell sorter using an antibody recognizing CD14. The vitality of the isolated monocytes was assessed by trypan blue exclusion and was generally above 95%.

Isolation of monocytes from buffy coats
For the experiments with H$_2$O$_2$, S100b, and bpV(phen) monocytes were isolated from buffy coats. The isolation was performed as described above.

Analysis of reversible PTP oxidation with ‘modified in-gel assay’
The protocol was adopted from Markova et al. In brief, monocytes (5x10$^6$ cells/assay) were isolated either from blood of DM and non-DM individuals or from buffy coats (as indicated). In the experiments with H$_2$O$_2$, monocytes were incubated with appropriate concentrations of H$_2$O$_2$ and for indicated time periods. Monocytes were first washed two
times with overnight degassed ice-cold PBS and then lysed in ice-cold buffer (degassed overnight) containing 50 mmol/L Hepes, pH 7.4, 150 mmol/L NaCl, 0.5% Nonidet P40, 1 mmol/L EDTA, 2mM EGTA, 20 μg/mL leupeptin, 1 μg/mL pepstatin, 200 KIE/mL aprotinin, and 1 mmol/L PMSF (protease inhibitors are added freshly). To detect reversibly oxidized PTPs, lysis was performed in the presence of 150 mmol/L sodium iodoacetate (‘modified in-gel assay’), similarly as described by Meng et al. Cell lysates were centrifuged at 13000 rpm for 15 minutes and aliquots of supernatant containing equal amount of protein were resolved on 10% SDS-PAGE containing 32P-labeled poly(Glu4Tyr) 2×10⁵ CPM/mL gel. Gels were treated as described and imaging was performed by exposure to high sensitivity films using an intensifying screen at -80°C. Images were scanned and analyzed using LAS-3000 software (Fuji Photo Film Co).

### Analysis of PTP activity

PTP activity was evaluated using Tyrosine Phosphatase Assay System (Promega). Monocytes were lysed using ice-cold buffer (degassed overnight) as above. After removal of insoluble debris, lysates were eluted through Sephadex G-25 to remove free phosphate. 2 μg of total protein from each sample was used to measure overall PTP activity following the manufacturer’s instructions. Measured absorbance was converted into amount of free phosphate after subtraction of corresponding control (lysate without addition of labeled substrate).

300 μg of total protein from each sample was used for PTP1B immunoprecipitation. First, lysates were pre-cleared with 25% anti-mouse IgG-Agarose. Subsequently, lysates were incubated with PTP1B antibody overnight followed by 2 hour incubation with 25% anti-mouse IgG-Agarose. Assay was performed with the immunoprecipitates. Measured absorbance was converted into amount of free phosphate after subtraction of
corresponding control (lysate after immunoprecipitation with omitted primary antibody without addition of labeled substrate).

**Monocyte chemotaxis**
Chemotaxis of freshly isolated monocytes was performed as described.\(^{15}\)

**Flow cytometry analysis**
The analysis was accomplished on a FACSCalibur flow-cytometer using the CellQuest software (Becton Dickinson). 100 µL of heparinized was used for staining. Staining with peridinin-chlorophyll-protein (PerCp)-conjugated anti-CD14 antibodies was used to define monocyte population. Staining for RAGE was performed subsequently following cell permeabilization with Triton X-100. Specific fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were subsequently applied.

For VEGFR-1 expression, isolated CD14+ monocytes were stained with VEGFR-1 antibody. Specific fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were subsequently applied.

**RT-PCR analysis of RAGE mRNA splice variants**
Total RNA was isolated with the RNeasy Mini Kit (Qiagen). First strand cDNA synthesis was performed with Omniscript reverse transcriptase (Qiagen). As a control the enzyme was omitted from the reaction. The presence of full length (F-RAGE), C-truncated (C-RAGE) and N-truncated (N-RAGE) RAGE splice variants was detected using the primers and PCR conditions as reported.\(^{4}\)
Western blot analysis of RAGE protein expression

Isolated monocytes (5×10^6 cells/assay) were centrifuged (5000 rpm, 4°C), washed with ice-cold PBS, pH 7.4 and subsequently solubilized in ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 10 mmol/L EDTA, 10% glycerol, 1% Trasylol (Bayer AG), 1 mmol/L phenylmethylsulfonyl fluoride; 1% Triton X-100. Cell lysates were centrifuged at 13000 rpm for 15 minutes and the protein concentration was estimated in the supernatant using the Bio-Rad protein assay.

Supernatants containing equal amounts of protein were heated in β-mercaptoethanol-containing sample buffer at 96°C for 5 minutes, and resolved using 7.5% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by a transfer onto nitrocellulose membranes (Amersham). Membranes were blocked with 5% semi-dry milk solution and incubated with specific primary antibodies. Antibodies recognizing human RAGE were used at a dilution of 1:8000. Horseradish peroxidase-conjugated secondary antibodies were added, and the specific proteins were detected using Supersignal chemiluminescence substrate (Thermo Fischer). The intensity of bands was quantified by scanning utilizing a LAS documentation system (Fuji Photo Film Co.).

Western blot analysis and immunostaining for Akt, p38 and ERK1/2

Isolated monocytes (5×10^6 cells/assay) were incubated in lysine-free RPMI 1640 medium containing 2% FCS for 10 minutes at 37°C in 2 mL tubes (Eppendorf). The cells were pretreated with 100 µmol/L Na3VO4 for 5 minutes. Subsequently, cells were stimulated with VEGF-A or PIGF-1 for 5 minutes at indicated concentrations. In experiments with H2O2, S100b or with bpV(phen), monocytes were incubated with appropriate concentrations and for indicated time periods.
Monocytes were centrifuged (5000 rpm, 4°C), washed with ice-cold PBS, pH 7.4, containing 100 µmol/L Na₃VO₄ and subsequently solubilized in ice-cold lysis buffer. Cell lysates were centrifuged at 13000 rpm for 15 minutes and the protein concentrations were determined from the supernatant using a dye reactive protein assay (Bio-Rad) according to the method of Bradford (595 nm). Supernatants containing equal amounts of protein were heated in β-mercaptoethanol-containing sample buffer at 96°C for 5 minutes and resolved on 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by a transfer onto nitrocellulose membranes (Amersham). Subsequently, membranes were blocked with 5% semi-dry milk solution and incubated with specific primary antibodies. Antibodies recognizing phosphorylated forms of Akt, p38 and ERK1/2 were used at a dilution of 1:1000. Horseradish peroxidase-conjugated secondary antibodies were added and the specific proteins were detected using Supersignal chemiluminescence substrate (Thermo Fisher). The intensity of bands was quantified by scanning utilizing an LAS documentation system. To monitor protein loading, blots were probed using 1:2500 dilutions of antibodies recognizing p38.

**Assessment of monocyte proliferation**

Monocytes were seeded at a density of 5x10⁵ cells/mL in 96-well plates in the presence or absence of PLGF-1 or VEGF-A at indicated concentrations. After 48 hours, the cell number was determined with: 1) Cell Titer 96 Non-Radiactive Cell Proliferation Assay (Promega) according to manufacturer’s instructions, followed by 2) cell counting using an automated cell counter CASY-TT (Scharfe System GmbH, Reutlingen, Germany) following the manufacturer’s instructions. All conditions were tested in triplicate in each experiment.
Assessment of monocyte apoptosis

Monocytes were seeded at a density of 5x10^6 cells/mL in 12-well plate. After adhesion (2 hours), medium was changed and monocytes were cultured for another 24 hours in the absence or presence of cycloheximide to induce apoptosis as previously described. VEGFR-1 ligands were added at the indicated concentrations together with cycloheximide.

References


Supplement figure legends

Supplement Figure 1. Expression of VEGFR-1 on primary human monocytes from non-diabetic and diabetic individuals. Representative FACS histogram plots of VEGFR-1 expression in non-diabetic (A) and diabetic (B) monocytes. Filled curve – staining with secondary antibody only, open curve – staining with primary antibody recognizing VEGFR-1 and secondary antibody. Expression of VEGFR-1 in non-diabetic and diabetic monocytes.

Supplement Figure 2. Effect of VEGFR-1 ligation on monocyte survival and proliferation. A. Effect of VEGFR-1 ligation on apoptosis of primary monocytes. Monocytes were cultured for 24 hours in the presence of cycloheximide and supplemented or not with the indicated concentrations of PlGF-1 and VEGF-A. Cleaved caspase-3 was assessed by Western blot. B, C. Effect of VEGFR-1 ligation on proliferation of primary monocytes. Monocytes were cultured for 48 hours in the presence or absence of the indicated concentrations of PlGF-1 and VEGF-A. Monocyte proliferation was evaluated using MTT dye conversion assay (B) and by subsequent cell counting (C). Results (mean) of 2 independent experiments.
Supplement Figure 1

A  nonDM

B  DM
Supplement Figure 2

A

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B

C

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- indicates the absence of treatment, + indicates the presence of treatment.